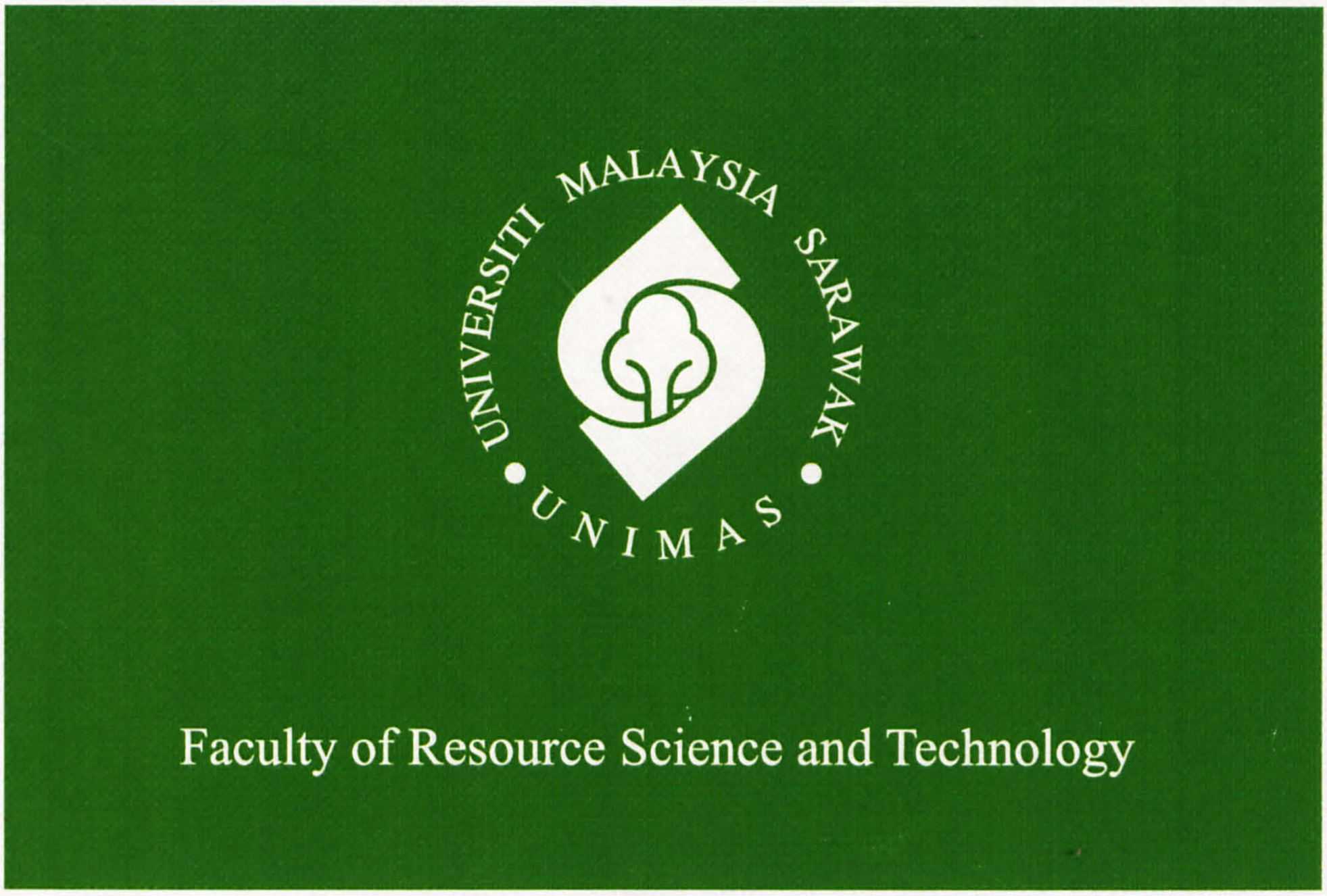

GENOTYPING OF INTERMEDIATE AND SAPROPHYTIC *LEPTOSPIRA* STRAINS ISOLATES FROM RATS, SOIL AND WATER IN SARAWAK USING BOX-PCR

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DECLARATION

I hereby declare that this Final Year Project report 2016 entitled "Genotyping of Intermediate and Saprophytic *Leptospira* Strains Isolates from Rats, Soil and Water in Sarawak using BOX-PCR" is based on my original work and effort except for the quotation and citation which have been dully acknowledged. I also declared that it has not been submitted for any degree at UNIMAS or other institutions of higher learning.

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LIST OF ABBREVIATIONS

BOX-PCR	-	BOX- Polymerase Chain Reaction
rep-PCR	-	repetitive extragenic palindromic- Polymerase Chain Reaction
EMJH	-	Ellinghausen McCullough Johnson Harris
dATP	-	deoxyadenosine triphosphate
dGTP	-	deoxyguanosine triphosphate
dCTP	-	deoxycytidine triphosphate
dTTP	-	deoxythymidine triphosphate
EDTA	-	Ethylenediaminetetraacetic acid
UPGMA	-	Unweight Pair Group Mathematical Averaging
rpm	-	Revolutions per minute
ml	-	Millilitre
µl	-	Microlitre
mg	-	Milligram
DNA	-	Deoxyribonucleic Acid
bp	-	base pair
kb	-	kilo base pair
°C	-	degree Celcius
spp.	-	species

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Genotyping of Intermediate and Saprophytic *Leptospira* Strains Isolates from Rats, Soil and Water in Sarawak using BOX-PCR.

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ABSTRACT

Leptospirosis is a globally re-emerging disease which can result to morbidity and mortality in both humans and animals. Leptospirosis is not new in Malaysia, yet the information and knowledge on this disease is still inadequate. This study aimed at genotyping intermediate and saprophytic *Leptospira* strains isolated from soil, water and rats in Sarawak by using BOX-PCR technique and to determine the genetic relatedness among intermediate and saprophytic *Leptospira* strains respectively. BOXA1R primer was able to produce BOX-PCR banding profiles with different fingerprint pattern among each of isolates. A dendrogram was constructed by using Dice coefficient similarity matrix and UPGMA based on fingerprint pattern obtained from the BOX-PCR profile. The phylogenetic analysis using PyElph software has characterized 29 intermediate isolates into 9 major clusters and 5 single isolates with calculated discriminatory index of 0.9434. Meanwhile, for the 41 saprophytic strains, they were clustered into 10 major clusters with 5 single isolates at the calculated discriminatory index of 0.932. The result of this study indicates that BOX-PCR can be used to study the genetic diversity and relatedness among the *Leptospira* strains based on the source and the location of isolation.

Keyword: *Leptospira,* BOX-PCR, genetic diversity, Sarawak.

ABSTRAK

Leptospirosis telah muncul sebagai salah satu penyakit yang harus dipandang serius kerana ia boleh menyebabkan morbiditi dan kematian kepada manusia serta haiwan. Walaupun leptospirosis sudah tidak asing lagi di Malaysia, pengetahuan serta maklumat berkaitan dengan penyakit ini masih tidak mencukupi. Tujuan utama kajian ini dijalankan adalah untuk mengklasifikasikan kumpulan 'intermediate' dan saprofitik Leptospira yang telah diambil daripada sampel tanah, air dan tikus di Sarawak menggunakan BOX-PCR disamping menentukan perkaitan antara genetik dalam kumpulan tersebut. Dengan menggunakan primer BOXA1R, jalur-jalur DNA telah berjaya dihasilkan dan telah menunjukkan kelainan antara satu dengan yang lain. Berdasarkan jalur-jalur DNA daripada BOX-PCR, pokok filogenetik telah dihasilkan dengan menggunakan persamaan matrik 'Dice coefficient' serta UPGMA. Pokok filogenetik yang telah dihasilkan menggunakan perisian PyElph telah mengklasifikasikan 29 'intermediate' Leptospira kepada 9 kumpulan utama dan 5 turunan tunggal dengan indeks pembezaannya 0.9434. Manakala bagi 41 saprofitik Leptospira, mereka telah diklasifikasikan kepada 10 kumpulan utama dan 5 turunan tunggal dengan indeks pembezaannya 0.932. Hasil kajian menunjukkan BOX-PCR boleh digunakan sebagai salah satu kaedah untuk memgkaji kepelbagaian dan perkaitan genetik dalam kalangan kumpulan Leptospira berdasarkan sumber dan lokasi Leptospira diambil.

Kata kunci: Leptospira, BOX-PCR, kepelbagaian genetic, Sarawak.

1.0 INTRODUCTION

Leptospira is gram-negative bacteria which cause leptospirosis. Leptospirosis has existed for a long time and this disease is considered as a typical disease that can be found even in Southeast Asia including Malaysia (Ahmed *et al.*, 2011; Thayaparan *et al.*, 2015). Leptospirosis can affect both humans and animals throughout the world resulting in morbidity and mortality (Thayaparan *et al.*, 2013). *Leptospira* has the ability to survive in a wide range of hosts including mammalians hosts where rats serve as the major carrier (Evangelista & Coburn, 2010). According to Balamurugan *et al.* (2013), *Leptospira* and its maintenance hosts appear to undergo adaptation to their environment thus, the preference and pathogenicity of these hosts also can change with time and geographical region. *Leptospira* can survive in moist soil and surface water for several months (Trueba *et al.*, 2004). Human infection can occur by direct contact with infected urine or more commonly by indirect exposure to the organisms in damp soil or water mainly through their skin and mucous membrane (Levett, 2001).

Leptospira is a highly complex bacteria that comprised twenty-two different species. They are further classified into three major groups, pathogenic, saprophytic and intermediate class based on their pathogenicity (Dietrich *et al.*, 2015). Research has been focused on the pathogenic strains meanwhile there is still lack of studies on the intermediate strains (Thayaparan *et al.*, 2015). Intermediate *Leptospira* strains tend to cause milder diseases in humans and animals but their virulence factor are still mysterious to the researchers (Chiriboga *et al.*, 2015). At the same time, saprophytic strain was reported as a free-living organism which does not cause harm as their presence is natural in environmental soil and water (Benacer *et al.*, 2013).

Leptospirosis has been reported in Malaysia since 1925 however, the knowledge on the epidemiological trends of this disease is still inadequate (Benacer *et al.*, 2016). Based on report by Zoonoses Disease Control Ministry of Health Malaysia (2015), Sarawak is one of the five states showing the high number of leptospirosis cases besides Kelantan, Selangor, Kedah and Terengganu. In Sarawak, the spread of the leptospirosis is believed caused mainly by bats and rodents (Thayaparan *et al.*, 2015). The climate condition in Sarawak provides a suitable environment for the *Leptospira* to survive. The exposure to the bacteria through occupation activities lead to high risk of leptospirosis infection (Benacer *et al.*, 2016). Leptospirosis is well known as a rural disease as the disposition of *Leptospira* within Rejang Basin community has been reported and the existence of *Leptospira* from periurban communities in Sarawak also have been documented (Su'ut *et al.*, 2011; Thayaparan *et al.*, 2015). Meanwhile, a study by Pui *et al.* (2015) highlighted the presence of pathogenic and intermediate *Leptospira* in the environment of Tanjung Datu National Park and Bako National Park. The presence of pathogenic and intermediate *Leptospira* indicated the potential for leptospirosis outbreak in these national parks. Therefore, the characterization of the *Leptospira* spp. will give a better understanding on the epidemiology pattern of the leptospirosis (Bourhy *et al.*, 2010). Hence it will help in diagnosis, treatment, and prevention on an outbreak.

Polymerase chain reaction (PCR) is a rapid and reliable method which is more preferable for genotype fastidious microorganism such as *Leptospira* spp. (Noubade *et al.*, 2002; Pui *et al.*, 2015). BOX- polymerase chain reaction (BOX-PCR) have shown high discriminatory power and easy to perform in typing bacteria (Syrmis *et al.*, 2004; Brusetti *et al.*, 2008). According to Brusetti *et al.* (2008), BOXA1R primer was targeting BOX element which is scattered in many bacterial genomes. Accordingly, the availability of the

BOX-PCR to genotype *Leptospira* spp. will give more benefit on ecological studies of *Leptospira* species.

There has been limited information on the circulation of *Leptospira* in Sarawak. Therefore, this study provides the knowledge on the distribution and genetic relatedness of the *Leptospira* strains isolated from different geographical areas in Sarawak. The objectives of the study were as follows:

i. To differentiate the genotype of saprophytic and intermediate *Leptospira* strains isolated from rats, soil and water collected from different areas in Sarawak using BOX-PCR.
ii. To determine the genetic relatedness of intermediate and saprophytic *Leptospira* strains respectively.

2.0 LITERATURE REVIEW

2.1 Morphology of *Leptospira*

Leptospires are Gram-negative, corkscrew-shaped bacteria which move actively in water and soil. They are different from the other Spirochaetes because of the presence of the end hooks (World Health Organization, 2003). Leptospires are mobile and their size is about 0.1 µm in diameter and 6-20 µm in length (Mohammed *et al.*, 2011). This bacterium is not easily stained and visualized by direct light thus, dark field microscopy or phase contrast is required for observation (Ahmed *et al.*, 2012).

Leptospires are obligate aerobe bacteria with an optimum growth temperature ranging from 28 °C to 30 °C. *Leptospira* does not resist drought or hypertonicity but they can live in alkaline environment up to pH 7.8. Leptospires have two periplasmic flagella with polar insertion located in the periplasmic space which is responsible for motility. They are known as flagella A and flagella B proteins. Flagella A constitute the flagellar sheath while flagella B constitute the core. Leptospires have double membrane structure consist of cytoplasmic membrane together with peptidoglycan cell wall that is closely associated. The membrane structure is also overlaid by an outer membrane (Mohammed *et al.*, 2011). The lipopolysaccharides located within the outer membrane of *Leptospira* are highly antigenic. The structural variations give rise to the large diversity for the serovars and serotypes (Sritharan, 2012).

2.2 Biology of Leptospires

Leptospira is a bacterium that can survive in mud, swamps, streams, liver and tissues of live or dead animals or even in the alkaline soil. Saprophytic species are naturally present in the environmental water and soil (Benacer *et al.*, 2013). Survival of pathogenic leptospires in the environment depends on several factors including pH, temperature and the presence of the inhibitory compound. They are sensitive to dryness, heat, and basic disinfectants thus will be killed when the temperature is greater than 50 °C (Mohammed *et al.*, 2011). Among the two pathogenic species, *L. borgpetersenii* only can survive within the mammalian host whereas *L. interrogans* can survive in the wet and moist environment outside the mammalian host (Sritharan, 2012). Optimal growth of pathogenic species is slow compared to saprophytic species. The pathogenic species require 28-30 °C at pH 7.2-7.6 for growing and only visible after the 3-4 weeks but, the saprophytic colonies is visible after only one week (Barer & Irving, 2012). The presence of saprophytic *Leptospira* does not cause diseases but commonly contaminates the unsterile materials.

2.3 Classification and Taxonomy of *Leptospira*

The taxonomy of *Leptospira* is complicated and has often changed over time until the recent use of molecular technique (Adler & Faine, 2006). Leptospires have been classified into serovars, where approximately 240 serovars were further divided into 24 antigenically related serogroups. This classification is achieved by using antisera specific to each serovar where these antisera react mainly against the lipopolysaccharide on the surface of *Leptospira* (Camahuali, 2009). Twenty-two species of *Leptospira* has been recognized based on genotypic classification where they are further grouping into three major classes, pathogenic, saprophytic and intermediate *Leptospira* (Barer & Irving, 2012; Dietrich *et al.*, 2015).

2.3.1 Serological Classification

Serological classification is a system that organizes individual bacterial strains of species into smaller groups based on their cell surface antigens. Serovar is the basic taxon and *Leptospira* genus have more than two hundred serovars with a triple-layered cell envelope, consists of an outer membrane containing long sugar polymer extensions called lipopolysaccharides (Slonczewski & Foster, 2011), a peptidoglycan cell wall and an inner membrane. Lipopolysaccharides are a major antigen recognized by the sera that infected humans and animals (Adler & Moctezuma, 2010). In serological classification, cross agglutinin absorption test (CAAT) or Microscopic agglutination test (MAT) is used to determine the *Leptospira* serovar however CAAT is a tedious and time-consuming technique (Romero *et al.*, 2009).

2.3.2 Genotypic Classification

The genotypic classification of *Leptospira* is based on the DNA hybridization and DNA relatedness of their species (Vaishnavi, 2013). According to the new genomic classification system, pathogenic species contains both pathogenic and nonpathogenic serovars as well as intermediate species such as *L.meyeri* (Morey *et al.*, 2006). The genotypic classification also has defined 22 species of *Leptospira* (Dietrich *et al.*, 2015) as shown in Table 2.3.2.1.

Table 2.3.2.1 Species of *Leptospira.*

Intermediate	Saprophytic	Pathogenic
L.inadai	*L.biflexa*	*L.interrogans*
L.fainei	*L.wolbachii*	*L.santarosai*
L.broomii	*L.vanthielii*	*L.weilii*
L.wolffii	*L.yanagawae*	*L.borgpetersenii*
L.licerasiae	*L.meyeri*	*L.noguchii*
	L. terpstrae	*L. kirschneri*
	L. idonii	*L. alexanderi*
		L. alstonii
		L. kmetyi
		L. mayottensis

The pathogenic *Leptospira* has the potential to cause diseases in animals and humans. Meanwhile, the saprophytic *Leptospira* is freely living and generally considered do not cause diseases because of the lack of genes for virulence (Vinetz, 2010). Intermediate *Leptospira* group has unclear pathogenicity which share genetic and growth characteristic of both saprophytic and pathogenic group (Vinetz, 2010). Intermediate *Leptospira* can grow better in culture. They are able to cause predominantly mild illnesses to human which can resolve by its own without the fatal complication (Lehmann *et al.*, 2014). Many studies have been conducted to understand the pathogenicity of the pathogenic, saprophytic and intermediate groups of *Leptospira.* Unfortunately, there is still limited information on the intermediate *Leptospira* group in comparison to pathogenic and saprophytic groups.

2.4 Geographical Distribution.

Human leptospirosis has a wide geographical distribution including Southeast Asia, Oceania, the Indian subcontinent, Caribbean and Latin America (Pappas *et al.*, 2008) except Antartica (The Center for Food Security & Public Health, 2013). *L. interrogans,* and *L. borgpetersenii* are most significant species for human infection in Asia (Cosson *et al.*, 2014). Human direct contact with the contaminated soil and water is verified as the main sources for human leptospirosis transmission (Lehmann *et al.*, 2014).

In Malaysia, the number of leptospirosis cases is influenced by flood result especially during rainy or monsoon season since water is the easier transmission medium for leptospirosis (Edre *et al.*, 2015). Since the monsoon season in East Malaysia is in between month October to March annually, the number of leptospirosis cases is inflating during this period (Benacer *et al.*, 2016). Furthermore, the high humidity and warm weather in Malaysia favors *Leptospira* to survive longer in the environment (Benacer *et al.*, 2016). Leptospirosis has been reported at picnic spot of Lubuk Yu, Maran, Pahang (Sapian *et al.*, 2012) and RSAT Army camp at Penrissen Batu 8, Kuching, Sarawak (Thayaparan *et al.*, 2013) where both cases are associated with contamination of the river by *Leptospira* (Thayaparan *et al.*, 2013). In addition, unhygienic environment with improper waste disposal system also contributed to the distribution of *Leptospira* spp. (Benacer *et al.*, 2013). Garbage that is not properly dispose can lure animals such as rats, birds and dogs which may be carriers for *Leptospira* spp.

2.5 Mode of Transmission

Transmission of leptospirosis can occur directly whereby contact with animal reservoirs blood or urine or indirectly through water contaminated with leptospires (Camahuali, 2009). *Leptospira* usually enters the body through the mucous membrane of the respiratory duct, abraded skin, ingested in contaminated food or water and inhalation of aerosols (Levett, 2001). They might be able to penetrate the intact skin that has been immersed for a long time in the contaminated water. *Leptospira* spp. is excreted in the urine of both acutely and chronically infected animals. In animals, *Leptospira* can be found in aborted or stillborn fetuses or normal fetuses or even in vaginal discharges after giving birth. Meanwhile, in humans they can be transmitted by sexual intercourse during convalescence or by breastfeeding (The Centre for Food Security & Public Health, 2013). Another uncommon route of exposure in people includes rodent bites or laboratory accidents (Williams & Barker, 2008). Human occupations such as mining, cleaning and farming as well as human activity like swimming or eco-tourism could increase the risk of exposure to leptospirosis infection (Cosson *et al.*, 2014; Thayaparan *et al.*, 2014).

2.6 The Genome of *Leptospira*

L. interrogans, L. borgpetersenii, and *L. biflexa* DNA strain have been completely sequenced (Barer & Irving, 2012). The genomes of *L. interrogans* serovar Lai vary from 4.7 Mb to 3.9 Mb of *L. borgpetersenii* serovar Hardjo (Tang *et al.*, 2014). All members of *Leptospira* genus that have been analyzed such as *L. interrogans* and *L. borgpetersenii* genome composed of two types circular chromosomes (Vke, 2011). The major circular chromosome carries most of the essential housekeeping genes while the small circular chromosome has a plasmid-like origin of replication (Tang *et al.*, 2014).

Meanwhile, the size of *L. biflexa* genome is 3.96 Mb and they consist of three chromosomes (Sritharan, 2012). The additional small circular chromosome will receive the transfer of important gene sets and seem to have co-evolved with the major chromosome. The identification of the genes that are related to motility, chemotaxis, adhesion and invasion cell in *Leptospira* genes reflects the ability of this organism to adapt to diverse environment stimuli (Ko *et al.*, 2009). Tang *et al.* (2014) also stated that *Leptospira* genome encodes about 2889 to 4033 coding sequences where 1500 of them are shared by all *Leptospira* spp. which are function as housekeeping genes related to DNA or RNA processing, bacterial metabolism, protein processing, maintenance of cell structure and cellular energy.

According to Picardeau *et al.* (2008), *L.biflexa* has more gene encoding environment-sensing and metabolic proteins compared to pathogenic *Leptospira* but, *L.biflexa* are lacks of orthologs for *LipL32, LipL41, HbpA, sphingomyelinases, Lig* protein, *LipL21 and LipL36* where they can be found in the pathogenic species (Sritharan, 2012). Repetitive element (IS element) also have been identified in *Leptospira* including IS *1500*, IS*1501* and IS*1533* (Lehmann *et al.*, 2014) where these element will help in mediate gene acquisition, inactivation or deletion of gene and rearrangement of large-scale *Leptospira* genome.

2.7 Treatment and Prevention of Leptospirosis

According to Disease Control Divison (2011), previously, the incidence of the leptospirosis is not well documented in Malaysia because of the lack of the attention on this disease. As the statistic of leptospirosis incidence in Malaysia increase, the disease become one of the notifiable disease and turn as one of the focus study for some medical institution in Malaysia. Leptospirosis is difficult to distinguish as their symptoms such as headache, myalgia, arthralgia, meningeal irritation, jaundice and gastrointestinal symptoms are quite similar with the other diseases. The symptoms can be experienced for about 1-2 weeks (Disease Control Divison, Ministry Health Malaysia, 2011).

Leptospirosis can only be treated if it is diagnosed early (Lim *et al.*, 2011) and it is commonly treated by antibiotic such as penicillin, doxycycline, macrolides, and aminoglycosides (Vke, 2011). The spreading of leptospirosis is commonly through infected animal such as rats. Therefore leptospirosis can be prevented by practicing a good personal hygiene (Lim *et al.*, 2011) and sanitation of the surrounding environment. Giving leptospirosis vaccine to pet such as dogs and cats also can help to preventing the spreading of *Leptospira*.

2.8 BOX-Polymerase Chain Reaction

BOX-PCR is a repetitive extragenic palindromic-PCR (rep-PCR) that uses the BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') as its primer. Masco *et al.* (2003) mentioned that BOX-PCR is a valuable tool for classifying and typing of a wide range of Gram-negative and several Gram-positive genera. This is because these techniques use outwardly facing oligonucleotide PCR primers which are complementary to interspersed repetitive sequences. Hence, it will enable the amplification of differently sized DNA fragments that are lying between these elements. BOX elements were the first repetitive sequence identified in Gram-positive *Streptococcus pneumoniae* (Martin *et al.*, 1992). Reading from 5'-3', BOX element consists of differentially conserved subunits namely *boxA, boxB, and boxC,* but only *boxA*-like subunit sequences appear highly conserved among diverse bacteria (Versalovic *et al.*, 1994). The DNA sequences of the BOX element is 154 bp long where *boxA* , *boxB* and *boxC* each consist of 59 bp, 45 bp and 50 bp long (Martin *et al.*, 1992). According to Knutsen *et al.* (2006), the nucleotide sequence of *boxA* element is 5'-TTATACTCTTCGAAAATCAAATTCAAACCACGTCAACGTCGCCTTGCCGTATAT ATGTGA-3'. Knutsen *et al.* (2006) also stated that, the nucleotide sequence of boxB element is 5'- CTGACTTCGTCAGTCCTATCTACAACCTCAAAACAGTGTTTTGAG-3'. On the other hand, the nucleotide sequence for *boxC* element is 5'-CAGCCTGCGGCTAGTTTCCTAGTTTGCTCTTTGATTTTCATTGAGTATTA-3'.

Since the BOX repetitive sequences are interspersed throughout the genome, BOX-PCR is potentially capable of simultaneously amplifying many DNA regions scattered in the bacterial genome (Brusetti *et al.*, 2008). BOX-PCR patterns do not affected by the culture age of the bacteria strain (Kang *et al.*, 2003). The fingerprinting output also can be easily analyzed by computer-assisted methods. According to Marques *et al.* (2008), BOX-PCR

has revealed the effectiveness in characterizing the *P. syringae* species, typing *Aeromonas* spp. strain and identification of species of *Ralstonia solanacearum.*

2.9 Characterization of Microbe using BOX-PCR Method.

A study conducted by Osek (2002) on the genetic relatedness of *Escherichia coli* 0157:H7 using BOX-PCR proved that this method is also a very sensitive method to reveal the intraserogroup genetic differences. By performing the random amplification of BOX DNA sequence on the 7 strains out of 372 strains, Osek found that PCR amplification of bacterial DNA from seven *E. coli* strains with BOX A1R resulted in the identification of four BOX types and randomly labels them as I, II, III and IV. Four out of seven strains are classified into BOX types I. Another three strains are classified into BOX- types II, III and IV respectively. The result of that study showed that despite the presence of different virulence marker genes and the origin of the strains, 100% of genetic relatedness for *E. coli* strains in BOX types I was observed and there are also high similarities of *E. coli* strains in BOX type I and type II. Comparison of the two *E. coli* strains from BOX type III and IV generates two separate clusters with a low percentage of genotypic similarities (Osek, 2002).

Another study made by Marques *et al.* (2008) using 120 bacterial strains belong to *Pseudomonas syringae sensu lato* groups and *P. viridiflava* also showed that pathovars belongs to the eleven genomospecies *Pseudomonas* including known species and unknown species can be separated at species level by BOX-PCR pattern. Through amplification of DNA banding pattern generated by BOX-PCR, Marques *et al.* (2008) successfully created a dendrogram tree which clustered all pathovars belong to one of the nine species design after Garden *et al.* (1999) under the Jaccard coefficient and UPGMA clustering method.